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Albumin coated liposomes: a novel platform for macrophage specific drug delivery

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Abstract

Here we report a new and efficient approach of macrophage specific drug delivery by coating liposomes with albumin. Activated albumin was reacted with liposomes containing polyethylene glycol (PEG) as hydrophilic spacers to create a flexible layer of covalently bound albumin molecules on the liposome surface. Albumin coated liposomes were taken up faster and more efficiently than uncoated liposomes by murine macrophages. Liposome uptake was significantly higher in macrophages as compared to other cell types tested (endothelial cells, fibroblasts, tumor cells), suggesting specificity for macrophages. In vivo, splenic macrophages phagocytosed BSA coated liposomes (BSA-L) at faster rates compared to conventional liposomes (L) and PEG liposomes (PEG-L). To prove the effectiveness of this new macrophage specific drug carrier, the bisphosphonates clodronate and zoledronate were encapsulated in BSA-L and compared with conventional liposomes. *In vitro*, treatment of macrophages with clodronate or zoledronate in BSA-L led to cytotoxic activity within a very short time and to up to 50-fold reduced IC_{50} concentrations. *In vivo*, clodronate encapsulated in BSA-L depleted splenic macrophages at a 5-fold lower concentration as conventional clodronate-liposomes. Our results highlight the pharmaceutical benefits of albumin-coated liposomes for macrophage specific drug delivery.

Introduction

Cells with phagocytic properties have been subject of investigation since their discovery by Metchnikoff more than a decade ago.[1] However, only the advent of modern technologies has made it possible to recognize the diverse roles macrophages play in an organism. Besides their homeostatic properties macrophages are the gate keepers of the immune system and they recognize and eliminate senescent and abnormal cells and generate signals that influence growth, differentiation and death of cells.[1-3] Several disorders like arthritis, atherosclerosis, asthma, inflammatory bowel disease and others origin from the pathological activity of macrophages, conditions also considered as chronic inflammation.[4-8] Microorganisms such as *M. tuberculosis*,[9,10] parasites[11,12] and viruses such as HIV[13-16] take advantage of macrophages as safe haven or powerful allies. In cancer, tumor associated macrophages contribute considerably to tumor growth and disease severity in many solid tumors.[17-19] Given the central role macrophages play in this variety of human diseases, effective targeting of drugs to these cells could be an astute strategy for efficacious prevention and treatment of infectious and inflammatory diseases and cancer.

A multitude of options to deliver drugs to macrophages have been developed in the past.[20] Most extensively studied are liposomes - nanosized unilamellar phospholipid bilayer vesicles.[21,22] Consequently, many drugs involved in macrophage-associated disorders have been studied using liposomes as carrier.

One of the most frequently reported application of macrophage specific liposome-mediated drug delivery are clodronate-liposomes that are successfully used to suppress macrophage activity by their depletion in various models of autoimmune diseases, transplantation, neurological disorders, viral and bacterial infections and cancer. Surprisingly, although effective in macrophage depletion, these liposomes have not been optimized for specific macrophage uptake, as they are composed of phosphatidylcholine and cholesterol, carry neutral charges and are either multilamellar or small unilamellar vesicles.[23-26] Hence, to enhance macrophage uptake, we introduced a non-specific modification by linking albumin to liposomes. Liposomes are recognized by macrophages by their opsonization, which is a process where serum proteins, in particular proteins of the complement system, attach to the liposome surfaces to facilitate recognition and phagocytosis.[27-33] Therefore, we reasoned that protein-coated and thus negatively charged liposomes would be phagocytosed more efficiently than conventional uncharged liposomes. We achieved this artificial opsonization by covalent attachment of albumin to the distal end of flexible poly (ethylene glycol) modified liposomes. Here we provide first proof of principle that albumin coated small unilamellar liposomes are a highly efficient drug delivery system for macrophages. Using fluorescence based approaches we noticed massive increase in uptake by macrophages when compared with conventional liposomes and with other cell types. Consequently, encapsulation of bisphosphonates in albumin coated liposomes led to a significant decrease of the cytotoxic dose and to more effective macrophage depletion *in vivo*.

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Key words: albumin coated liposomes, macrophage, specific targeting, drug delivery, bisphosphonates.

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Contributions: RS, CV, SK, study conception and manuscript writing; CV, SK, experiments and data analysis; SK, manuscript revising.

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Materials and Methods

Chemicals

Soy phosphatidylcholine (SPC) was from L. Meyer, Hamburg, GE and 1.2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(maleimide (polyethylene glycol)2000) (DSPE-PEG-Mi) from Avanti Polar Lipids (Alabaster, Alabama, USA). Cholesterol (Chol), human serum albumin (HSA), N-ethylmaleimide and cysteine were from Fluka, Switzerland. D,L α-tocopherol (α-toc) and mannitol from Merck (Darmstadt, Germany). 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiI) was from Molecular Probes (Invitrogen, Carlsbad, CA, USA). Hydroxylamine chloride, murine (MSA) and bovine serum albumin (BSA) were from Sigma-Aldrich (St. Louis, MO, USA). Clodronate was a gift from Farchemia SrL (Treviglio, Italy) and zoledronate a gift from Novartis International (Basel, Switzerland). N-succinimidyl-S-acetyl-thioacetate (SATA) was prepared as described.[29]

Liposome preparation

All liposomes were prepared by freeze-thawing and high pressure extrusion as

described.[24] Lipid compositions were as follows: (i) Conventional liposomes (L), SPC 104 µmol/mL (80 mg/mL), Chol 20 µmol/mL (8 mg/mL), α-toc 1.04 µmol/mL (0.45 mg/mL); (ii) PEG-liposomes (PEG-L) and albumin coated liposomes (BSA-L, MSA-L, HSA-L), SPC 104 µmol/mL, Chol 20 µmol/mL, α-toc 1.04 µmol/mL, DSPE-PEG-Mi 2.1 µmol/mL (6 mg/mL); (iii) Conventional clodronate (CL) liposomes, SPC 130 µmol/mL (100 mg/mL), Chol 25 µmol/mL (10 mg/mL), α-toc 1.3 µmol/mL (0.56 mg/mL) and (iv) PEG clodronate (PEG-CL) liposomes and BSA coated clodronate (BSA-CL) liposomes, SPC 130 µmol/mL, Chol 25 µmol/mL, α-Toc 1.3 µmol/mL, DSPE-PEG-Mi 2.6 µmol/mL (7.3 mg/mL). For fluorescent liposomes DiI was added at 0.35 µmol/mL (0.3 mg/mL).

Lipids were dissolved in methanol/methylene chloride (1:1, v/v) and solvents removed by evaporation at 40°C (Rotavapor, Büchi Labortechnik AG, Flawil, Switzerland). Dried lipid films were dispersed in phosphate-mannitol buffer (PB-Man, 20 mM phosphate, 230 mM mannitol, pH 7.4), supplemented with 66.25 mg/mL clodronate or 4 mg/mL zoledronate for the bisphosphonate liposomes. Liposomes were obtained by three cycles of freeze-thawing in liquid nitrogen and 40°C water, followed by repetitive extrusion through NucleporeTM membranes (Sterico AG, Dietikon, Switzerland) of 400 nm (3x) and 100 nm (8x) pore size using a Lipex™ extruder (Northern Lipids Inc., Burnaby, BC, Canada). For the clodronate liposomes (3 and 4) extrusion was done with 400 nm filters (8x) and non-encapsulated biphosphonate was removed by extensive dialysis (Spectrapore tube, 12-14 kD mol.wt. cut-off) using PB-Man as dialysis buffer (1:100, v/v). All preparations were sterile filtrated (0.45 µm Millex-HV filters, Millipore, Billerica, MA, USA). Liposome size and homogeneity were measured by dynamic laser light scattering (Nicomp 370, Nicomp Corp., Santa Barbara, CA, USA). The ζ-potential was determined with a Malvern Zetasizer 3000 HAS (Malvern Instruments, Malvern, UK) and bisphosphonate encapsulation assessed by liquid scintillation counting (Tri-Carb, Canberra Packard Int., Zurich, Switzerland).

Albumin coupling

Albumins were coupled to the liposomes as described.[34] Thiolation was performed by modification of earlier described methods.[35] SATA (1 mg) dissolved in dimethylformamide (100 µL) was added per ml albumin solution (10 mg/mL) and incubated 1h at RT in HEPES buffer (10 mM HEPES, 145 mM NaCl, 2 mM EDTA, pH 7.4). Unreacted SATA was removed by dialysis (Spectrapore tube, 12-14 kD cut-off) at 4°C overnight. Thioacetylated albumin was deacetylated by addition of hydroxylamine (1M, 100 µL/mL BSA, 30 min). Albumin coupling to freshly prepared liposomes was done by incubation at equal volumes at RT for 8 h. Addition of N-ethylmaleimide (1:60 mol/mol) stopped the reaction. Unreacted maleimide residues were blocked by addition of excess cysteine as described before.[36] Unbound albumin was removed by gel filtration on Sephadex G-100 columns. Protein content was measured using the Bradford assay according to the manufacturer's instructions (Bio-Rad Laboratories GmbH, Munich, Germany).

Cell lines and cell culture

Murine macrophages (RAW 247.6, ATCC TIB-71) and Lewis lung carcinoma (LLC, ATCC CRL-1642) cells were cultivated in RPMI 1640 with L-glutamine (10% FBS, 10'000 U/mL penicillin, 10 mg/mL streptomycin). Murine NIH 3T3 fibroblasts and pancreatic islet endothelial MS1 (ATCC CRL-2279) cells were cultivated in DMEM with 4.5 mg glucose/L (10% FBS and antibiotics). All cells were maintained at 37°C/5% CO^2 in a humidified incubator.

Flow cytometry and fluorescence microscopy

Cells were seeded at a density of 25,000 cells per well in 24-well plates and grown in RPMI medium for 48 h. Cells were incubated with medium either containing: (i) DiI-labeled L; (ii) DiI-labeled PEG-L or (iii) DiI-labeled BSA-L at concentrations of 10 (8 ng), 50 (20 ng) or 250 (200 ng) nmol SPC/mL liposomes in 300 µL medium for different incubation times (10 min, 30 min, 1 h, 3 h and 24 h). Then, cells were washed three times with PBS. For macrophages, the cell layer was gently scraped in PBS using a cell scraper to facilitate detachment of the cells. For the other cell types, cells were harvested by trypsinization and washed once with PBS. Cells were analyzed by flow cytometry (CyAn 9 ADP Beckman Coulter, Nyon, Switzerland) to distinguish the uptake between the three types of liposomes. Cells were measured for DiI fluorescence and forward and side scatter were used to gate living cells. Liposome uptake was calculated by dividing the mean log of DiI fluorescence of liposome-treated viable cells by the mean log of DiI fluorescence of untreated control cells. For fluorescence microscopy cells were washed in PBS after the indicated incubation times, fixed with formaldehyde (3% in PBS, AppliChem GmbH, Darmstadt, Germany) and incubated with DAPI (2 µg/mL in PBS, Roche Diagnostics GmbH, Mannheim, Germany) for 10 min in order to stain the cell nuclei. The uptake of liposomes to the different cell types was examined using an Olympus OBS IX81 microscope (Olympus, Tokyo, Japan).

Cell viability

Cell viability was evaluated using the resazurin method.[37] Cells (25,000/well) in 24-well plates were grown for 30 h. Medium containing increasing concentrations of 1-1000 µM for CL, PEG-CL and BSA-CL or 0.1-10 µM for ZL, PEG-ZL and BSA-ZL was added for 1 h, 3 h, 24 h or 48 h. Then, cells were washed 3x with PBS and the 1 h and 3 h treatments were additionally incubated with RPMI for 23 h or 21 h, respectively, to allow the bisphosphonates to take effect. Resazurin (0.5 mL in medium) was added and after 4 h incubation at 37°C fluorescence was measured at 590 nm emission with 540 nm excitation wavelength in a SpectraMax M5/M5e Reader (Molecular Devices Corp., Sunnyvale, CA, USA). Cytotoxicity (IC_{50}-values) was determined by graphic extrapolation by plotting drug concentration against percentage of viable cells and taking untreated cells as 100% viability. Empty liposomes were not toxic to cells (data not shown). All measurements were carried out twice in duplicates.

Animal studies

Analysis of macrophage depletion in spleens was done in C57Bl/6 mice (3/group) by i.v. injection of 50 µL of DiI-labeled L or BSA-L. After 1.5 or 6 h the spleens were removed and immersed in ice cold DMEM (10% FBS, 4.5 mg glucose/L, 1% antibiotics) followed by mashing on a 70 µm cell strainer (Becton Dickinson Labware, Le Pont de Claix, France) to create a single cell suspension. After centrifugation (2000 rpm, 5 min) the pellet was resuspended and filtrated (35 µm nylon mesh filter, Beckton Dickinson), followed by 10 min treatment with red blood cell lysis buffer (Pharm Lyse™, BD Pharmingen, San Diego, CA, USA), centrifuged and re-suspended in FACS buffer (2% FCS in PBS). The CD16/32 Fc blocking antibody (1:300, v/v, BioLegend, San Diego, CA, USA) was added to decrease the background signal. Cells were incubated with the F4/80-APC antibody (1:200 in FACS buffer) for 30 min on ice and immediately analyzed (CyAn™ 9 ADP). To study depletion of macrophages, C57Bl/6 mice (3/group) received 0.2 or 1 mg CL or BSA-CL by i.p. injection or empty BSA-L as controls. After 24 h the spleens were removed and cell suspensions obtained as described above. Percentage of macrophage depletion was determined by flow cytometry of F4/80 stained cells.

Statistical analysis

Comparisons among different liposome preparations were made by the unpaired Student's t-test using the GraphPad Prism 5.03 software. Differences were termed statistically significant at $P<0.05$ and data expressed as mean±SD.

Results

A schematic representation of conventional liposomes L, poly(ethylene glycol) liposomes PEG-L and albumin coated liposomes BSA-L is shown in Figure 1.

The structural changes of BSA coated liposomes are summarized in Table 1. The mean diameter of L was 96.8±1.7 nm with a nearly neutral ζ-potential. Addition of DSPE-PEG2000-Mi at 2 mol%, - in contrast to 7.5 mol% used for conventional long circulating PEG-liposomes[38] - increased the diameter by 10 nm and decreased the ζ-potential by 5 mV as reported previously.[38]

Covalent coupling of BSA to PEG-L increased liposome size by about 40 nm and decreased the ζ-potential to -10 mV, confirming that negatively charged albumin was attached on the outer liposome surface. Liposome size changed slightly after storage up to 60 days at 4°C (Table 1). Cell incubation results were identical with liposomes stored for 60 days, indicating good liposome stability (data not shown). The observed size increase of PEG-L is the direct consequence of pegylation, which leads to a coating thickness of 5 nm.[38] BSA molecules are negatively charged at physiological pH and have either a heart-like shape, which can be approximated by a triangular shape,[39] a prolate ellipsoid[40] or an oblate ellipsoid,[41] depending on the method of structure analysis used. Calculation of the BSA concentration on the liposome surface based on the data obtained from the Bradford protein assay resulted in an average of 64±3 µg BSA/µmol total lipids, which corresponds to 100-130 BSA molecules attached to one liposome calculated as decribed.[36] These results are in accordance with those reported by Thöle *et al*.[42]

Encapsulation of the bisphosphonates resulted in similar sizes as observed with empty liposomes (Table 2). The encapsulation rate of 30-33 % (18±2.5 mg/mL for clodronate and 1.25±0.3 mg for zoledronate) was determined by radioactive trace labeling (data not shown). Here, it is noteworthy that the mean diameter of clodronate liposomes after repetitive extrusion through 400 nm membranes resulted in a range of 187±88 nm which is considerably smaller than expected. Clodronate probably interacts with the lipid bilayer in a way that a condensation effect occurs. To study the preferential uptake RAW macrophages were treated with DiI-labeled L, PEG-L and BSA-L and subsequently analyzed by fluorescence microscopy and flow cytometry. As shown in Figure 2, BSA-L were taken up more efficiently by the macrophages in a time and concentration dependent manner. At each time point the amount of BSA-L taken up was significantly higher than that of L and PEG-L. Importantly, there was no difference in uptake between BSA-L, HSA-L, and MSA-L (Figure 2A, C) suggesting that the higher uptake of albumin coated liposomes was not due to the antigenicity of a foreign protein (BSA or HSA on murine macrophages) but rather due to the albumin coating. This permits to choose the albumin independently of the species studied, without provoking immune responses that could be induced by antigenic albumin. Consequently, for the following experiments, BSA-L were used.

The flow cytometry results (Figure 2D) showed that macrophage uptake of BSA-L was about 17-times higher after 1 h incubation and remained 4-times higher after 24 h as compared to L. Compared to PEG-L uptake of BSA-L was 53-times higher after 1 h incubation and 9 times higher after 24 h incubation. These differences are concentration independent since no change in uptake was observed at liposome concentrations of 10 and 50 nmol SPC/mL (data not shown). The results suggest that macrophages phagocytose BSA-L at faster rates than the control liposomes L and PEG-L. Another aspect highlighted by these results is that PEG-L uptake by macrophages is significantly lower as compared to L, confirming earlier findings.[43]

Examination of macrophages exposed to DiI-labeled BSA-L at a higher magnification (Figure 2B) revealed that DiI was uniformly distributed on the surface and in the cytoplasm of the macrophages suggesting that higher uptake is due to stronger surface binding of BSA-L and higher phagocytic activity. To evaluate the specificity of BSA-L towards macrophages, three other cell lines were analyzed in a comparable set of experiments; cancer cells (Lewis lung carcinoma, LLC), fibroblasts (NIH 3T3) and endothelial cells (MS-1). Uptake of DiI-labeled liposomes was significantly lower in these cells when compared to the macrophages (Figure 3). After 3 h or 24 h incubation DiI-labeled BSA-L uptake increased on macrophages between 40- and 220-fold (Figure 2D), compared to a significantly lower

Table 1. Liposome size and ζ-potential of L, PEG-L and BSA-L. (n=3 for mean particle size and n=5 for ζ-potential measurements).

Liposome	Mean diameter (nm)	Mean diameter after 30 days (nm)	Mean diameter after 60 days(nm)	Zeta potential (mV)
L	96.8±7	98.2±2.2	97.6±2.8	-1±0.6
PEG – L	111.3±59	110.6±2.6	111.5±2.5	-5.5±0.7
BSA – L	151.1±01	153.3±5.9	158.5±3.9	-10.7±.4

Table 2. Liposome size, encapsulation rate and cytotoxicity (IC_{50} in µM, see also Figure 5) of bisphosphonates in liposomes (n=3).

Liposome	Mean diameter (nm)	Encapsulation rate (%)	IC_{50} 3 h	IC_{50} 24 h	IC_{50} 48 h
CL	186.5±88	30.9 ±2.5	nd	nd	nd
PEG-CL	184.2±52	30.3±1.0	nd	nr	620
BSA-CL	215.8±80	30.3±1.0	200	40	nd
ZL	184.2±54	33.7±1.4	nr	50	4
PEG-ZL	177.5±26	32.8±0.3	nr	>50	5.6
BSA-ZL	213.7±97	32.8±0.3	3.6	1	0.225

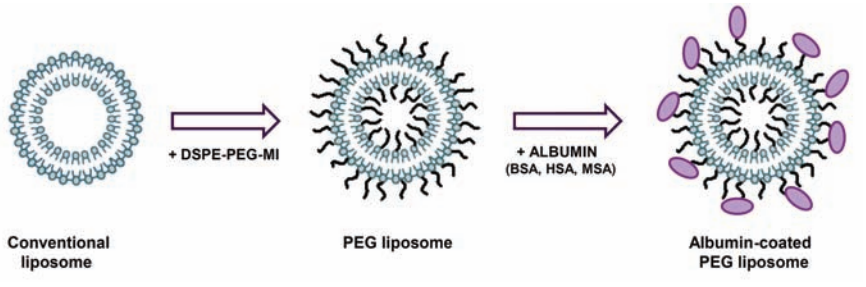

Figure 1. Schematic representation of conventional liposomes L, poly(ethylene glycol) liposomes PEG-L and albumin coated liposomes BSA-L. Albumin coating is achieved by the reaction of activated thiolated albumin with maleimide groups located at the terminal ends of the poly(ethyleneglycol) chains on the liposome surface.

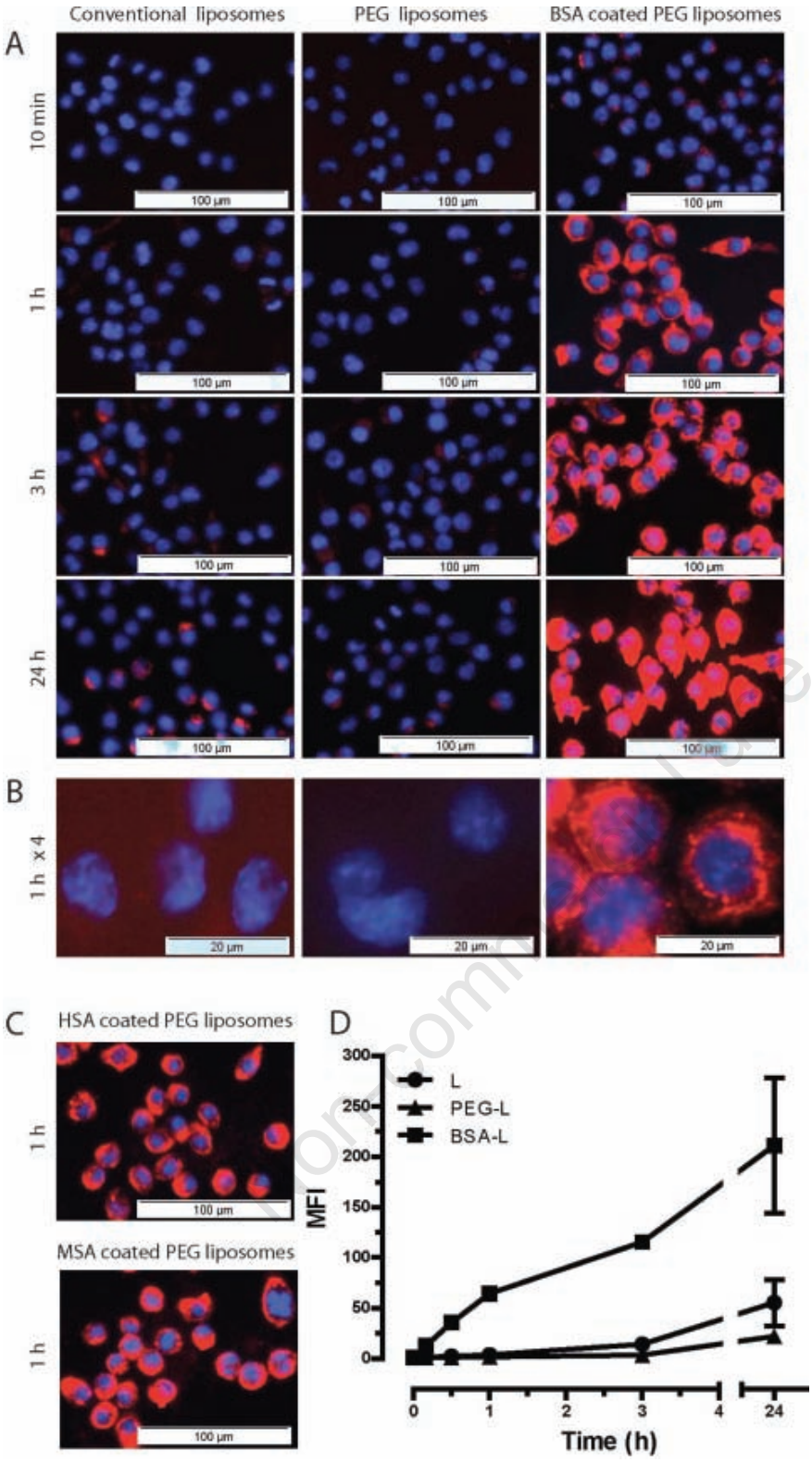

Figure 2. ***In vitro*** **uptake of DiI-labeled liposomes by macrophages. (A) Fluorescence microscopy images of macrophages incubated with 250 nmol SPC/mL DiI-labeled liposomes at different time points. (B) Uptake after 1h incubation at higher magnification (640 x). (C) Uptake of DiI-labeled HSA-L and MSA-L after 1h. Red is fluorescence of DiI and blue is fluorescence of DAPI. (D) Quantification of the cellular uptake of DiI-labeled liposomes by macrophages by flow cytometry (MFI, mean fluorescence intensity). Liposome uptake was calculated by dividing the mean log of DiI fluorescence of liposome-treated cells by the mean log of DiI fluorescence of untreated cells.**

1- and 12-fold uptake for the other cells.

These results confirm the target specificity of the albumin-coated liposomes towards macrophages. When analyzed by flow cytometry slight differences of liposome uptake between the cell lines were observed, probably by virtue of the high sensitivity of the method. The flow cytometric analysis also showed low uptake of L by any other cell line, in comparison to the macrophages which was further decreased when BSA-L and PEG-L were used (Figure 3).

Coating the liposome surface with inert, biocompatible polymers such as PEG interferes with the ability of liposomes to interact with target cells.[44,45] Rather, such polymer coating at saturating concentrations is used to prolong the blood circulation time of liposomes.[38,43-45] The evaluation of our results suggests that albumin coated liposomes have a 2-fold advantage over uncoated liposomes, on one hand they are preferred nanoparticles for macrophages and on the other hand they show distinctly lower uptake by other cell types. The in vivo characteristics of the uptake of DiI-labeled L, PEG-L and BSA-L were assessed in spleens after intravenous injection (Figure 4). PEG-L were not used as control since several studies including ours showed that surface-grafted PEG reduce opsonization leading to prolonged blood circulation and reduced macrophage uptake.[43-45] The spleen is the peripheral reservoir of myeloid cells that is constantly replenished by bone marrow myeloid progenitors.[2] The analysis of spleen cells revealed that there was no significant difference between BSA-L and L concerning the percentage of targeted macrophages as reflected by the comparable percent values of F4/80 positive cells ranging from 1.61-2.35% of all viable splenocytes (Figure 4A, upper right quadrant). Rather, it seems that macrophages phagocytose albumin-grafted liposomes at a highly faster rate compared to other liposome types. Ninety minutes after liposome injection

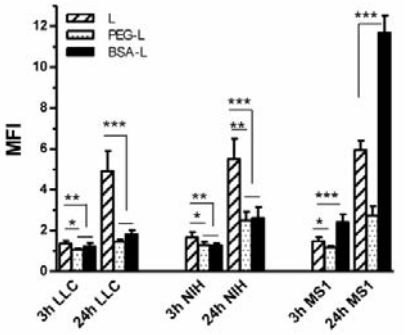

Figure 3. ***In vitro*** **evaluation of the uptake of DiI-labeled liposomes by LLC, NIH 3T3 and MS-1 cells by flow cytometry. MFI, mean fluorescence intensity; *, P<0.05; **, P<0.01; ***, P<0.001.**

the population of F4/80+ macrophages which had taken up DiI-labeled liposomes had already reached 35.14% (Figure 4A, cells in box in upper right quadrant) with the BSA-L, while no such population was found after injection of L. After 6 h, uptake of L had reached 6.1%, whereas it had decreased from 35.14-27.5% with BSA-L. This result indicates that 6 h after administration of BSA-L macrophages were already saturated while the rate of phagocytosis of L was still increasing. The decrease of the DiI-positive macrophage population after BSA-L treatment between 90 min and 6 h can possibly be explained by exocytosis of liposomes, whereas exchange of the lipophilic dye DiI between macrophages and other cells can be excluded due to the high membrane stability of the dye.[46] The bar graph shown in Figure 4B recapitulates the macrophage uptake results of three individual experiments. These data correlate well with the results from the in vitro studies and confirm the *in vivo* efficiency of BSA-L specifically targeting macrophages. Hence, we can assume that uptake of albumin coated liposomes *in vivo* by macrophages is faster and occurs at higher avidity compared to L. Higher uptake of BSA-L by macrophages is indicative of enhanced delivery of encapsulated drugs. Thus, we tested the cytotoxic effect of clodronate and zoledronate containing liposomes on macrophages. In Figure 5, dose-response curves are presented and the extrapolated IC_{50}-values are summarized in Table 2. BSA-CL were highly toxic to macrophages showing effects already after 1 h and reaching an IC_{50} of 200 µM after 3 h. When compared to CL, the BSA-L were 15.5 times more effective in the 24 h assay. Correspondingly, BSA-ZL liposomes were 50- and 25-times more toxic after 24 h and 48 h, respectively. The effect of the zoledronate-liposomes is comparable to results reported by Shmeeda *et al*. describing strong cytotoxic effects of folate targeted liposomes on various cell types expressing folate receptors at similar IC_{50} concentrations.[47]

Thus, our results show that BSA-L deliver the encapsulated bisphosphonates at faster rates and more efficiently to macrophages, causing cytotoxic effects at shorter incubation times as compared to controls. This is particularly important in the case of conventional clodronate-liposomes, since the quantity of the administered drug to deplete macrophages is considerable and in the range of the LD_{50} for mice (5.54 mM or 2 mg/20 g mouse weight).[24] CL are widely used to deplete macrophages.[23-26] Therefore, we tested the macrophage-depleting efficacy of BSA-CL in mouse spleens and compared it with CL. Application of 1 mg of BSA-CL caused 67.6±3.5% depletion of the F4/80+ population of spleen macrophages after 24 h, whereas after CL treatment depletion reached only 36.2±9.4%. At 0.2 mg the depletion efficiency of BSA-CL (43.7±3.5%) was similar to that of 1 mg CL (Figure 6). These results further confirm that BSA-L are not only preferred for phagocytosis by macrophages but that they also deliver drugs more efficiently and consequently deplete macrophages at lower clodronate concentrations.

Discussion

In this study we prepared albumin-coated liposomes with remarkably increased uptake properties on macrophages compared to conventional liposomes. Such macrophage specific liposomes represent an interesting platform to transport drugs or other compounds efficiently to macrophages, thus opening new possibilities to treat diseases where macrophages are involved. Liposomes are mainly recognized by macrophages equally as other nanoparticulate carriers and pathogens by their opsonization, a process where serum proteins, in particular those of the comple-

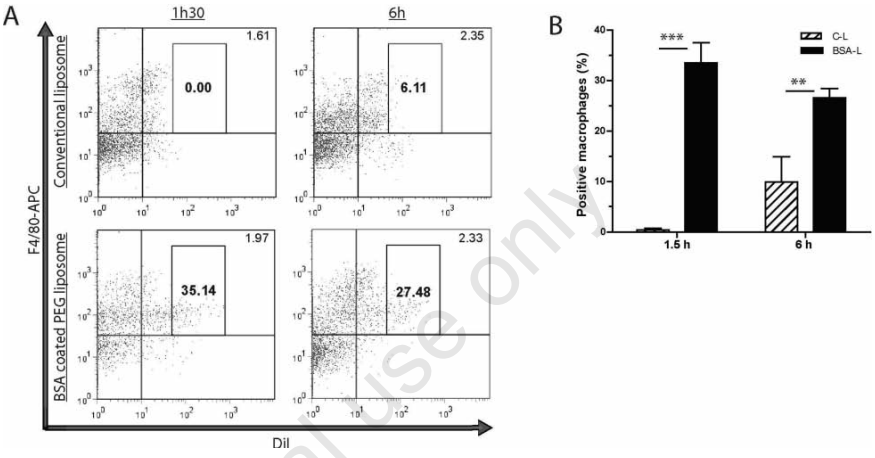

Figure 4. *In vivo* evaluation of the cellular uptake of DiI-labeled liposomes by spleen macrophages by flow cytometry. In A, a representative logarithmic plot of F4/80+ macrophages (F4/80-APC) versus DiI-labeled liposomes (DiI) is shown. B summarizes the mean percentage of F4/80+ macrophages which had a 5 times higher DiI fluorescence intensity (cells in box in upper right quadrant in A) compared to all macrophages selected in the upper right quadrant. DiI-liposome uptake was quantified by determining the percentage of fluorescent viable macrophages. Non specific fluorescence was eliminated by gating the signal of non treated mice (logPE=10) and percentages of F4/80+ macrophages were determined by eliminating the signal of unstained samples. The uptake of the two types of liposomes by macrophages was evaluated by gating the F4/80+ macrophages, which had a 5-fold higher fluorescence (logPE=50) than all positive fluorescent macrophages. The bars represent the mean percentage of viable macrophages ± SD, n=3; **, P<0.01; ***, P<0.001.

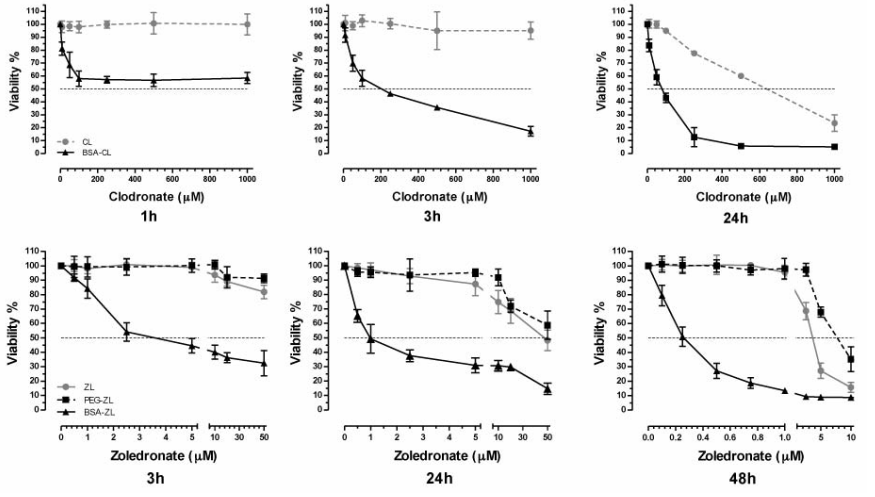

Figure 5. Viability of macrophages after treatment with CL and BSA-CL (upper panel) and ZL, PEG-ZL and BSA-ZL (lower panel) after 1 h, 3 h and 24 h or 48 h drug incubation.

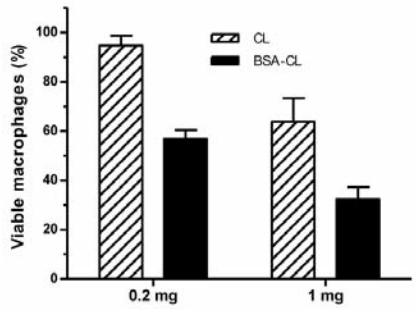

Figure 6. ***In vivo*** **evaluation of the depletion of splenic macrophages with CL and BSA-CL by flow cytometry (n=3). To study the depletion of macrophages C57Bl/6 mice (n=3) were treated by intraperitoneal injection of 0.2 or 1 mg of CL or BSA-CL or an equivalent number of empty BSA-L as controls. After 24 h the spleen was removed and cell suspensions were obtained. Macrophages were identified by gating the F4/80-APC channel as described in Figure 4.**

ment system, attach to their surfaces to facilitate recognition and phagocytosis.[27-33] Moreover, the degree of liposome binding and subsequent ingestion by macrophages depends on a number of factors including lipid composition, vesicle type, size and surface properties. Small unilamellar liposomes deliver drugs more effectively than larger uni- or multilamellar liposomes and charged liposomes associate more effectively to cells and deliver their content more efficiently than neutral liposomes.[21,22,48,49] Finally, coating liposome surfaces with proteins or cell surface specific ligands such as Fc-receptor, complement, folate, fibronectin, lipoproteins, mannosyl and galactosyl receptors and others significantly promote uptake of liposomal content by macrophages.[21,22,50-55] However, many of these strategies have shortcomings such as system complexity, production costs and restricted applications and none of them has been routinely exploited to increase macrophage uptake of liposomes for therapeutic interventions. Since the modification of albumin with a bispecific-coupling molecule as SATA and its subsequent reaction with maleimide-modified PEG-lipids are straightforward reactions that occur in aqueous media, we argue that the preparation of albumin coated liposomes is an easy procedure. Potential disadvantages encountered with the preparation of larger batches remain to be investigated.

Therefore, it appears that enhancement of liposome binding and uptake by macrophages could be achieved by interference with protein adsorption and reactions with the complement system. It has been shown that neutral liposomes are poor activators of the complement system as compared to negatively charged vesicles.[30,56] Besides charge, liposome size and concentration play also an important role in complement activation.[28,57]

Consequently, we reasoned that protein-coated and thus negatively charged unilamellar liposomes of intermediate sizes would be phagocytosed more efficiently than conventional liposomes. We achieved this *artificial opsonization* by covalent attachment of albumin to PEG-modified liposomes.

Albumin is constantly synthesized in the liver and its homeostasis is maintained by a balanced catabolism occurring in all tissues. Most albumin is degraded in muscle, liver and kidney.[40] Albumin itself is not taken up by macrophages and it is only upon its covalent linking to nanoparticulate vesicles such as liposomes that macrophage uptake occurs. Thus, possible mechanisms that improve and accelerate the uptake of albumin coated liposomes are that the protein may accelerate opsonisation or that binding of the liposomes to the macrophage cell surface is facilitated and therefore phagocytosis occurs at higher rates as compared to uncoated liposomes. However, the exact mechanisms remain to be elucidated.

More recently, albumin-liposome conjugates have been studied in order to develop long circulating drug carriers.[58,59] In these reports it was shown that introduction of albumin on the surface of conventional PEG-liposomes prolonged circulation in plasma compared to PEG-liposomes. However, in contrast to our formulations, in these studies albumin was directly grafted onto the lipid surface of liposomes containing PEG at the conventional amounts used for long circulating liposomes. Thus, the albumin molecules directly attached to the liposome surface were covered by the PEG chains, which might prevent or slow down liposome opsonization. In this study, bisphosphonates were used as prototype drugs, however it can be assumed that other drugs such as protein inhibitors or DNA and RNA (e.g. siRNA, microRNA and anti-microRNA) based therapeutics or nanozymes encapsulated in albumin-liposomes and targeted to macrophages will have similar pharmaceutical advantages.[60-63]

In summary, in this study a new nanodrug carrier platform for specific macrophage delivery, - albumin coated liposomes -, was developed and evaluated *in vitro* and *in vivo*. These surface coated liposomes are characterized by a significantly improved uptake and specificity towards macrophages, a lower uptake rate by non myeloid cell types and an enhanced accumulation in splenic macrophages, as compared to conventional and pegylated liposomes. Our results indicate that albumin coated liposomes represent a promising platform for macrophage specific drug delivery for the administration of bisphosphonates and other drugs and compounds.

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